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SUSCEPTIBILITY

Antimicrobial Susceptibility of 260 *Clostridium botulinum* Type A, B, Ba, and Bf Strains and a Neurotoxigenic *Clostridium baratii* Type F Strain Isolated from California Infant Botulism Patients

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ABSTRACT Infant botulism is an infectious intestinal toxemia that results from colonization of the infant large bowel by *Clostridium botulinum* (or rarely, by neurotoxigenic *Clostridium baratii* or *Clostridium butyricum*), with subsequent intraintestinal production and absorption of botulinum neurotoxin that then produces flaccid paralysis. The disease is often initially misdiagnosed as suspected sepsis or meningitis, diagnoses that require prompt empirical antimicrobial therapy. Antibiotics may also be needed to treat infectious complications of infant botulism, such as pneumonia or urinary tract infection. Clinical evidence suggests (see case report below) that broad-spectrum antibiotics that are eliminated by biliary excretion may cause progression of the patient's paralysis by lysing *C. botulinum* vegetative cells in the large bowel lumen, thereby increasing the amount of botulinum neurotoxin available for absorption. The purpose of this antimicrobial susceptibility study was to identify an antimicrobial agent with little or no activity against *C. botulinum* that could be used to treat infant botulism patients initially diagnosed with suspected sepsis or meningitis, or who acquired secondary infections, without lysing *C. botulinum*. Testing of 12 antimicrobial agents indicated that almost all California infant botulism patient isolates are susceptible to most clinically utilized antibiotics and are also susceptible to newer antibiotics not previously tested against large numbers of *C. botulinum* patient isolates. No antibiotic with little or no activity against *C. botulinum* was identified. These findings reinforce the importance of promptly treating infant botulism patients with human botulism immune globulin (BIG-IV [BabyBIG]).

KEYWORDS *Clostridium baratii*, *Clostridium botulinum*, human botulism immune globulin (intravenous), anaerobes, antibiotic susceptibility, botulinum toxin, botulism, infant botulism

Infant botulism (IB) is an acute symmetric descending flaccid paralysis that occurs in infants 12 months of age or less that results from botulinum neurotoxin (BoNT) produced by *Clostridium botulinum* (and rarely *Clostridium butyricum* or *Clostridium baratii*). Illness develops after spores of these organisms are swallowed and reach the infant's large intestine, where they germinate, multiply, and release BoNT into the intestinal lumen. Intraluminal toxin is absorbed via lymphatics into the circulation and transported to the neuromuscular junction, where it binds presynaptically, blocks acetylcholine release, and produces flaccid paralysis (1–3).

Although more than 40 years have elapsed since the initial recognition of infant

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botulism, "suspected sepsis" remains the most common admission diagnosis of patients who are later confirmed by laboratory studies to have infant botulism (1, 2). Infants initially diagnosed with possible sepsis or suspected meningitis require prompt empirical treatment with parenteral broad-spectrum antibiotics, some of which reach the lumen of the colon via biliary excretion. Additionally, patients with laboratory-confirmed IB may require antimicrobial therapy for community-acquired or nosocomial infections. A common concern in clinical management is that lysis of *C. botulinum* in the large intestine by such antibiotics may worsen the patient's paralysis by increasing the amount of BoNT available for absorption (4, 5).

Hence, identification of broad-spectrum antibiotics that have limited or absent bactericidal activity against *C. botulinum* would provide clinicians with better therapeutic choices. Two antimicrobial susceptibility studies of *C. botulinum* were reported more than 35 years ago (6, 7). Fifteen years ago, the antimicrobial susceptibilities of six neurotoxigenic *Clostridium butyricum* type E strains obtained from six patients with intestinal toxemia botulism were reported (4).

This study sought to determine whether California *C. botulinum* infant botulism patient isolates (i) were sensitive to ceftriaxone (because it is commonly used), (ii) were resistant to other cephalosporins or fluoroquinolones, and (iii) displayed the same susceptibility patterns seen with previously tested antimicrobials (6, 7). The intent was to identify an antimicrobial agent that could be used to treat secondary infections in IB patients that would not lyse *C. botulinum* vegetative cells temporarily residing in the large intestine, the same question that Fenicia et al. investigated with neurotoxigenic *C. butyricum* type E (4).

We report here the susceptibility of 260 strains of *C. botulinum* and a single strain of neurotoxigenic *C. baratii* isolated from California infant botulism patients, as well as three additional strains of neurotoxigenic *C. baratii* that were not isolated from California IB patients, to a variety of antibiotics.

Case report. A previously healthy 3.5-month-old female infant with a 2-week history of constipation was admitted to a South Carolina hospital, presenting with marked fatigue and a urinary tract infection. Her urine culture grew an *Enterobacter* sp., and a 7-day course of intravenous cefotaxime was started at admission. By hospital day 3 (HD 3), she had developed ptosis and general weakness. A stool specimen tested at the National Botulism Reference Laboratory, Centers for Disease Control and Prevention (Atlanta, GA) contained BoNT/A and *C. botulinum* type A, thereby establishing the diagnosis of infant botulism. The patient was not treated with human botulism immune globulin (BIG-IV [BabyBIG]) because at the time of her illness, the product was only available if administered within 7 days of hospital admission (2, 3). On HD 17, the patient was treated with a single dose of intravenous ceftriaxone for suspected pneumonia caused by a *Staphylococcus* species isolated from a tracheal aspirate. Within 24 h, she developed complete paralysis that required intubation and ventilatory support for the subsequent 27 weeks (i.e., more than half a year). In the months following her treatment with ceftriaxone, she experienced recurrent urinary tract infections, respiratory syncytial virus pneumonitis, and several failed attempts at extubation. However, with continuous meticulous supportive care alone (i.e., no tracheostomy and no gastrostomy), she recovered completely and was discharged to home after a total hospital stay of 9.5 months.

Ceftriaxone is excreted by the kidney into the urine and by the liver into the biliary tree and small intestinal lumen (8). Approximately 33 to 67% of ceftriaxone is eliminated in the bile, and the remainder is eliminated in the urine (8). This case provided the impetus to identify an antibiotic(s) that could be used to treat nosocomial or community-acquired infections in infant botulism patients without risking possible lysis of intestinal *C. botulinum* with concomitant release of intracellular BoNT.

RESULTS

The percentages of *C. botulinum* type A ($n = 156$), *C. botulinum* type B ($n = 89$), and *C. botulinum* type Ba ($n = 12$) strains inhibited across a range of MICs by the following

TABLE 1 Percentage of *C. botulinum* type A strains inhibited across a range of MICs of 12 antimicrobial agents

Antimicrobial agent tested	Cumulative % *C. botulinum* type A strains ($n = 156$) with MIC (μg/ml)[a] of:									
	≤0.50	≤1	≤2	≤4	≤8	≤16	≤32	≤64	≤128	≤256
AMC[b]	96	99	100							
AMP	98.7	99.3					100[c]			
PEN	99						100[c]			
FEP	1	3	25	62	86	92	100			
CTX	8	38	83	98	99	100				
CRO	11	46	89	99	100					
CXM	1	13	81	97	99	100				
GEN							4	7	10	100
MXF	87	97	99	100						
NAL					3	8	20	37	50	100
SXT[d]	65	71	76	78			100			
VAN			2	8	61	97	99	100		

[a]MIC values determined by Etest method (AB Biodisk, Solna, Sweden).
[b]Concentration represents the concentration of amoxicillin; amoxicillin and clavulanic acid were present at a ratio of 2/1.
[c]A single highly resistant strain tested positive for beta-lactamase production by the chromogenic nitrocefin disk test (Hardy Diagnostics, Santa Maria, CA).
[d]Concentration represents concentration of trimethoprim; trimethoprim and sulfamethoxazole were present at a ratio of 1/19.

12 antibiotics are shown in Tables 1 to 3: ampicillin (AMP), penicillin (PEN), amoxicillin-clavulanic acid (AMC) (2/1), cefuroxime (CXM), cefotaxime (CTX), ceftriaxone (CRO), cefepime (FEP), moxifloxacin (MXF), gentamicin (GEN), trimethoprim-sulfamethoxazole (SXT) (1/19), nalidixic acid (NAL), and vancomycin (VAN). The MICs for *C. botulinum* type Bf ($n = 2$) and *C. baratii* type F ($n = 4$) strains were also determined.

Penicillin (with one exception) demonstrated the greatest inhibitory activity of the antibiotics tested. Ninety-nine percent of all *C. botulinum* type A and B strains were inhibited by ≤0.50 μg/ml PEN. Similar results were achieved with ≤1.0 μg/ml AMP and ≤1.0/0.5 μg/ml AMC. Most *C. botulinum* type Ba strains and both *C. botulinum* type Bf strains were inhibited by ≤0.50 μg/ml PEN and AMP and by ≤2.0 μg/ml AMC.

Unexpectedly, a single strain of *C. botulinum* type A demonstrated high-level resistance to both PEN and AMP, each having an MIC of 32 μg/ml. However, this strain was susceptible to AMC, a beta-lactam–beta-lactamase inhibitor combination, with MICs of 0.75/0.375 μg/ml (9). This penicillin-resistant type A strain was found to produce beta-lactamase using the chromogenic nitrocefin disk test (Nitrocef; Hardy Diagnostics, Santa Maria, CA).

Collectively, cephalosporin antibiotics demonstrated higher MICs than did PEN or AMP. CRO was the most active cephalosporin against all *C. botulinum* strains and against *C. baratii* type F strains, followed in descending order by CTX, CXM, and FEP. More than half of all *C. botulinum* type A and B strains were inhibited by an MIC of

TABLE 2 Percentage of *C. botulinum* type B strains inhibited across a range of MICs of 12 antimicrobial agents

Antimicrobial agent tested	Cumulative % *C. botulinum* type B strains ($n = 89$) with MIC (μg/ml)[a] of:									
	≤0.50	≤1	≤2	≤4	≤8	≤16	≤32	≤64	≤128	≤256
AMC[b]	84	100								
AMP	98	99	100							
PEN	99	100								
FEP	3		31	70	85	89	100			
CTX	16	53	92	98	99		100			
CRO	19	69	92	100						
CXM		13	65	92	100					
GEN					1		7	11	13	100
MXF	87	97	99	100						
NAL					2	7	10	17	23	100
SXT[c]	70	78	85	91	92	93	100			
VAN			3	15	34	90	99	100		

[a]MIC values determined by Etest method (AB Biodisk, Solna, Sweden).
[b]Concentration represents the concentration of amoxicillin; amoxicillin and clavulanic acid were present at a ratio of 2/1.
[c]Concentration represents concentration of trimethoprim; trimethoprim and sulfamethoxazole were present at a ratio of 1/19.

TABLE 3 Percentage of *C. botulinum* type Ba strains inhibited across a range of MICs of 12 antimicrobial agents

Antimicrobial agent tested	Cumulative % *C. botulinum* type Ba strains ($n = 12$) with MIC (μg/ml)[a] of:									
	≤0.50	≤1	≤2	≤4	≤8	≤16	≤32	≤64	≤128	≤256
AMC[b]	75		92	100						
AMP	75		100							
PEN	92	100								
FEP				2	42		100			
CTX		25	50	75			100			
CRO	33	50	67	92	100					
CXM			58	92	100					
GEN							25	50	58	100
MXF	67	83	92		100					
NAL					8	17	33		50	100
SXT[c]	58									100
VAN			8	33	75	100				

[a]MIC values determined by Etest method (AB Biodisk, Solna, Sweden).
[b]Concentration represents the concentration of amoxicillin; amoxicillin and clavulanic acid were present at a ratio of 2/1.
[c]Concentration represents concentration of trimethoprim; trimethoprim and sulfamethoxazole were present at a ratio of 1/19.

≤2.0 μg/ml of CRO, CTX, or CXM. FEP was the least active of the cephalosporins, with just 50% of type A and type B strains being inhibited at concentrations between 2 and 4 μg/ml. In general, all bivalent *C. botulinum* type Ba and Bf and *C. baratii* type F strains were more resistant to the cephalosporins than were *C. botulinum* type A and B strains. More than half of the type Ba strains were inhibited only by >8 μg/ml FEP. This evident increase in FEP MIC for the 12 type Ba strains may reflect in part the issue that relatively fewer bivalent Ba than type A and B strains were tested (Table 3). The two *C. botulinum* type Bf bivalent strains were inhibited by 3.0 and 12.0 μg/ml FEP, respectively, and 2 of 4 *C. baratii* type F strains were completely resistant to FEP at ≥32 μg/ml (low-range Etest strip). The other 3 cephalosporins evaluated inhibited the 2 *C. botulinum* type Bf and the 4 *C. baratii* type F strains at an MIC of ≤6.0 μg/ml.

MXF was the only fluoroquinolone evaluated in this study. MXF was active against all strains of *C. botulinum* and *C. baratii* type F tested. Of the *C. botulinum* type A and type B strains, 87, 97, 99, and 100% were inhibited at 0.5, 1, 2, and 4 μg/ml, respectively. The MIC at which more than 50% of *C. botulinum* type A, B, and Ba strains were inhibited by MXF was ≤0.50 μg/ml. All strains of *C. baratii* type F were inhibited by ≤0.50 μg/ml MXF. The 2 strains of *C. botulinum* type Bf had MXF MICs of 1.0 and 4.0 μg/ml.

The MIC of SXT for 76% of *C. botulinum* type A and 85% of *C. botulinum* type B strains was ≤2/38 μg/ml when tested on blood Mueller-Hinton II (BMH) agar (Tables 1, 2, and 4). Twenty-two *C. botulinum* type A strains and 12 *C. botulinum* type B strains had SXT MICs of ≤0.008/0.152 μg/ml when grown on BMH but had SXT MICs of ≥32/608 μg/ml when grown on both formulations of WCA. One type A strain had an MIC of ≥32/608 μg/ml when grown on WCA supplemented with 5% lysed horse blood (LHB) but had an MIC of 3/57 μg/ml when grown on WCA supplemented with thymidine phosphorylase (TP).

TABLE 4 Percentage of California *C. botuli*num strains inhibited across a range of MICs of SXT compared to percent inhibition reported by Swenson et al.

Study	Cumulative % *C. botulinum* strains inhibited by SXT with MIC (μg/ml)[a] of:						
	≤0.50	≤1	≤2	≤4	≤8	≤16	≤32
Swenson et al. (6) ($n = 224$)[b]	13	17	19		100		
This study ($n = 260$)[c]	63	69	75	78	79	80	100

[a]Concentration represents concentration of trimethoprim; trimethoprim and sulfamethoxazole were present at a ratio of 1/19.
[b]Study included *C. botulinum* types A to G, at $n = 109$, 65, 5, 1, 32, 11, and 1, respectively, isolated from human feces, wounds, and a variety of food sources. MICs were determined by the agar dilution method on WCA supplemented with 5% LHB.
[c]Study included *C. botulinum* types A and B, Ba, Bf, and *C. baratii* type F, at $n = 156$, 89, 12, 2, and 1, respectively. All strains isolated from feces of individual infant botulism patients. MICs were determined by Etest method on BMH.

No differences in antibiotic activity were found between strains from different geographical regions of California (data not shown).

DISCUSSION

At clinical presentation, patients with infant botulism often appear to have sepsis or meningitis and hence are treated empirically with broad-spectrum and combination antimicrobial therapy. Recommended empirical triple-antibiotic therapy includes VAN and AMP plus CTX, CRO, or FEP (10, 11). Patients with laboratory-confirmed IB may also require antimicrobial therapy for secondary or community-acquired pulmonary or urinary tract infections, and such therapy often includes CTX or the combinations of CRO and GEN or AMP and GEN, respectively (10, 11). CRO, which is widely used in the United States for pediatric patients, has a broad antimicrobial spectrum. However, CRO did not exist in 1980 when the first extensive *in vi*tro antibiotic susceptibility study of *C. botulinum* was done (6). Clinical observations suggest that CRO, which is excreted by the biliary system into the small intestine (8), may lyse *C. botulinum* vegetative cells with the release of BoNT and exacerbation of illness (4).

California *C. botulinum* patient isolates were found to be highly susceptible to PEN. Nearly all strains, both types A and B, were inhibited by less than 0.50 μg/ml PEN. However, a single strain of *C. botulinum* type A demonstrated substantial resistance to both AMP and PEN and was found to produce beta-lactamase. Mazuet et al. reported a similar strain also isolated from an IB patient in France (12). Additionally, *C. botulinum* was sensitive to the 4 cephalosporins tested, some of which have not been previously tested against large numbers of *C. botulinum* strains. CRO had the greatest activity of the cephalosporins tested; all *C. botulinum* type A strains were inhibited at $\leq$8 μg/ml, and all *C. botulinum* type B strains were inhibited at $\leq$4 μg/ml. These findings suggest that IB patients treated with a cephalosporin, especially CRO, may be at increased risk of developing more extensive and sustained paralysis (4).

MXF was included in this study as a representative of the fluoroquinolones, an antibiotic class that has not previously been tested against large numbers of *C. botulinum* strains. Although MXF is generally contraindicated for treating pediatric patients because of possible cartilage damage and possible prolongation of the QT interval, it can be used under certain circumstances for treating serious infections if no other effective treatments are available (13). Like the cephalosporins, all *C. botulinum* and neurotoxigenic *C. baratii* type F strains tested were sensitive to MXF.

No differences in susceptibilities between *C. botulinum* types A and B strains were observed. The antimicrobial susceptibilities of bivalent *C. botulinum* type Ba and Bf strains were comparable to those of the monovalent type A and B strains (Tables 1 to 3). Also, no differences in susceptibilities were found between strains from different geographical regions of California. Furthermore, the MICs of most antibiotics previously studied, including those for GEN, NAL, and VAN, were similar to those in our findings (Tables 1 to 3). Like Dezfulian and Dowell (6) and Swenson et al. (7), we found that GEN was relatively inactive against CA strains of *C. botulinum* and *C. baratii*. However, the possible clinical appeal of GEN use is tempered by its synergism with BoNT in potentiating neuromuscular blockade (14).

A notable result of our study is the apparent discordant MIC data for SXT compared to those previously reported by Dezfulian and Dowell (6) and Swenson et al. (7). In their 1980 study of *C. botulinum* antibiotic susceptibility, Swenson et al. reported that most type A and B strains were resistant to the trimethoprim-sulfamethoxazole (SXT) combination (7). Dezfulian and Dowell reported similar findings (6). Unexpectedly, we found the opposite. In our study, the percentage of *C. botulinum* type A and B strains inhibited by SXT over a range of MICs indicated that these strains were mostly susceptible to this antimicrobial. In accordance with standard interpretive guidelines and the Etest manufacturer's instructions, the MIC of SXT was interpreted at 80% inhibition when diffuse growth was present (15). Distinct zones of growth inhibition were evident for many of the strains inhibited by a low SXT MIC, thereby precluding the possibility of misinterpretation due to diffuse or sparse growth.

Swenson et al conducted their antimicrobial susceptibility study using the agar dilution method and Wilkins-Chalgren agar (WCA) (Table 4). To prevent inhibition of SXT by thymidine, they supplemented WCA with 5% lysed horse blood, which is a natural source of thymidine phosphorylase, the enzyme that converts thymidine to thymine (5). It is possible that the lysed horse blood added by Swenson et al. (7) did not provide enough thymidine phosphorylase in the media. The residual thymidine may have then inhibited the SXT, thereby allowing *C. botulinum* to grow and give the appearance of resistance to SXT. In contrast, in our susceptibility studies, we used a commercially prepared BMH agar known to contain inconsequential amounts of sulfonamide inhibitors, the specifics of which are proprietary (16).

Our initial MIC data found that most California *C. botulinum* strains were inhibited by ≤2/38 μg/ml SXT when grown on BMH (Tables 1 to 4). However, a subset of strains was found to be resistant to 32/608 μg/ml SXT when grown on WCA. All media passed quality control testing for sulfonamide inhibition (15). Monthly quality control testing of media in our laboratory using a standardized suspension of *C. botulinum* results in smaller colonies on botulinum selective medium than the colonies grown on nonselective egg yolk agar. This is also true for wild-type strains of *C. botulinum*. These findings further suggest that *C. botulinum* is partially inhibited by SXT. It is unknown if the Etest method used to determine the MIC of SXT in our study played a role or if other factors, such as the homogeneity of the source of the California strains, is contributory. SXT was the only drug we tested in which our results did not correlate with those previously reported (6, 7). Nonetheless, these discordant observations may warrant further investigation.

In conclusion, our study was unable to identify an antimicrobial agent to which *C. botulinum* and neurotoxigenic *C. baratii* strains were consistently resistant that could then be safely used to treat concomitant bacterial infections in patients with infant botulism. We sought to identify such an antibiotic to avoid the possibility that intraluminal lysis of *C. botulinum* vegetative cells in the colon might increase subsequent botulinum toxemia. Patients treated with BIG-IV before starting antimicrobial therapy avoid this risk because of the large toxin-neutralizing capacity and long half-life of BIG-IV (2). Treatment with BIG-IV before administration of antibiotics (or as soon as possible thereafter) enables physicians to select the optimal antibiotic regimen for the patient without concern for susceptibility of *C. botulinum* to the antibiotics prescribed.

MATERIALS AND METHODS

Strains. A total of 260 botulinum neurotoxin-producing strains of *Clostridium* isolated from California patients hospitalized with infant botulism was studied, consisting mainly of *C. botulinum* type A ($n = 156$) and proteolytic *C. botulinum* type B ($n = 89$) strains. Proteolytic *C. botulinum* type Ba bivalent strains (those that produce both BoNT/A and BoNT/B) ($n = 12$), proteolytic *C. botulinum* type Bf bivalent strains ($n = 2$), and a neurotoxigenic *Clostridium baratii* type F strain ($n = 1$), all isolated from California IB patients, were also tested. Three additional neurotoxigenic *C. baratii* type F strains isolated from 2 non-California infant botulism patients and one CA adult patient were also studied. All isolates came from the Infant Botulism Treatment and Prevention Program (IBTPP) culture collection.

Strain selection criteria. To ensure statewide geographic coverage of isolates tested, strains from all counties in California identified as the county of residence at the onset of illness were tested ($n = 42$ [CA has 58 counties]). All strains from each county ($n = 15$) that had 4 or fewer infant botulism patients were included. For those counties ($n = 24$) that had more than 4 but fewer than 60 patients, 30% of the patient strains were tested. For those counties ($n = 3$) with 60 or more patients, 20% of the patient strains were tested. All *C. botulinum* type Ba and Bf strains and *C. baratii* type F strains in the collection at the time of the study were tested. In the more densely populated counties of California, both *C. botulinum* type A and type B strains cause infant botulism. The number of toxin type A and toxin type B strains selected for testing from each county having more than 4 cases was determined by the percentage of cases caused by each toxin type. For those counties in which >4 infant botulism cases occurred, the oldest case isolate and the most recent case isolate were selected for testing, with the remaining isolates chosen to be evenly distributed by year of isolation.

Susceptibility testing. Etest strips (AB Biodisk, Solna, Sweden) were used to determine the MICs of the following 12 antimicrobial agents: amoxicillin-clavulanic acid (AMC) (2/1), ampicillin (AMP), cefepime (FEP), cefotaxime (CTX), ceftriaxone (CRO), cefuroxime (CXM), gentamicin (GEN), moxifloxacin (MXF), nalidixic acid (NAL), penicillin (PEN), trimethoprim-sulfamethoxazole (SXT) (1/19), and vancomycin (VAN).

Susceptibility testing was performed on 150-mm-diameter blood Mueller-Hinton II (BMH) agar plates supplemented with 5% sheep blood (Hardy Diagnostics, Santa Maria, CA). This medium was chosen for

its ability to support the luxurious growth of *C. botulinum* and for its low thymidine concentration, a necessary condition for accurately testing bacterial susceptibility to SXT (15). Large quantities of BMH and Etest strips (each spanning just two lot productions) were purchased to avoid possible fluctuations in MIC values as a consequence of lot-to-lot product variation. The BMH agar and Etest strips underwent in-house quality control testing to verify performance characteristics that included testing for possible presence of sulfonamide inhibitors in the BMH agar (15).

All isolates were subcultured from frozen stocks to culture tubes containing prereduced chopped meat glucose starch broth (CMGS; produced in-house) and incubated at 35°C for 72 h in a Bactron IV anaerobic chamber (90% N_2, 5% CO_2, and 5% H_2 gas atmosphere; Sheldon Mfg., Cornelius, OR), in which all further experimental manipulations occurred. One hundred microliters of actively growing CMGS broth culture was streaked for isolation on 4% egg yolk agar plates (produced in-house) and incubated at 35°C for 48 h. Isolated colonies were emulsified in physiologic saline and adjusted to a turbidity equal to 0.5 (±0.05) McFarland standard using a Vitek densitometer (bioMérieux, Hazelwood, MO). BMH agar plates prereduced in the anaerobe chamber overnight were inoculated with the adjusted bacterial suspensions using standard techniques (AB Biodisk Etest technical guide 1B: Susceptibility testing of anaerobes Solna, Sweden) (9, 17). Each isolate was inoculated onto two BMH agar plates, and 6 different Etest strips were applied to each plate, thereby testing a total of 12 antibiotics per isolate. Plates were examined after approximately 24 h of incubation for a confluent lawn of growth and for distinct elliptical zones of bacterial inhibition before interpreting the MIC of each antibiotic. Less than 3% of the isolates required extended incubation (always less than 48 h) to reach visible confluent growth. The MIC values for each antibiotic were interpreted in accordance with Etest manufacturer guidelines (AB Biodisk Etest technical guide 1B: Susceptibility testing of anaerobes, Solna, Sweden). A single *C. botulinum* type B strain could only be cultured on Schaedler agar supplemented with 5% sheep blood and 100 units/liter thymidine phosphorylase (TP). There were no significant differences in MIC values for this single isolate compared to all other type B isolates tested on BMH agar.

Trimethoprim-sulfamethoxazole testing. Botulinum selective medium (BSM) that contains trimethoprim and sulfamethoxazole enables selective isolation of *C. botulinum* from human fecal flora (18). In addition, previous studies reported that *C. botulinum* type A and proteolytic *C. botulinum* type B strains exhibited resistance to trimethoprim and sulfamethoxazole (6, 7). However, initial results using BMH agar indicated that most strains in the IBTPP collection were susceptible to SXT. For this reason, we tested 36 strains (24 type A and 12 type B) that showed susceptibility to SXT when grown on BMH and compared their sensitivities to SXT when grown on Wilkins-Chalgren agar (WCA).

Two formulations of WCA were prepared in-house and compared to BMH agar for determining the MIC of SXT. Dehydrated Wilkins-Chalgren medium (Oxoid, Basingstoke, England) was rehydrated per the manufacturer's instructions, and agar (Oxoid) was added to a final concentration of 2.5%. The first preparation of WCA was supplemented with 5% lysed horse blood (LHB) (7), and the second preparation was supplemented with 100 units/liter thymidine phosphorylase (TP), the amount present in BSM (18). Both formulations of WCA were poured in 150-mm-diameter petri dishes to a standard depth of 4 mm and were quality control tested for sulfonamide inhibition (15).

Twenty-four *C. botulinum* type A and 12 *C. botulinum* type B strains were cultured on WCA that contained 5% LHB and on WCA that contained 100 units/liter TP. Both formulations of WCA passed quality control testing to verify the absence of sulfonamide inhibition. Media that yield a zone of growth inhibition diameter of ≥20 mm for *Enterococcus faecalis* ATCC 29212 around a disk impregnated with SXT are considered acceptable for testing sulfonamides (15). Interestingly, in quality control testing, BMH agar demonstrated a zone of inhibition of 40 mm, while for both formulations of WCA, the zone of inhibition was only 23 mm. Additionally, the WCA that contained LHB had heavier diffuse growth around the SXT disk (but still within 80% inhibition) than did the WCA that contained 100 units/liter TP.

MIC interpretation. Interpretive standards for anaerobic Gram-positive organisms exist only for PEN, AMP, and AMC (9). There are no MIC susceptibility breakpoints for the other 9 antibiotics when tested against Gram-positive anaerobes. For this reason, testing results are reported here as the percentage of strains inhibited by each antibiotic across a range of MICs.

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